Nomination of the whole blood pyrogen test for extension to non-endotoxin pyrogens

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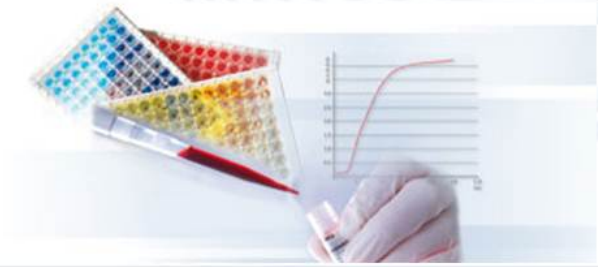
Pyrogen (bacterial and fungal contamination) testing still consumes more than 300.000 rabbits per year

Limulus assay is restricted to Gram-negative endotoxin, disturbed by many substances and does not reflect human pyrogen potency

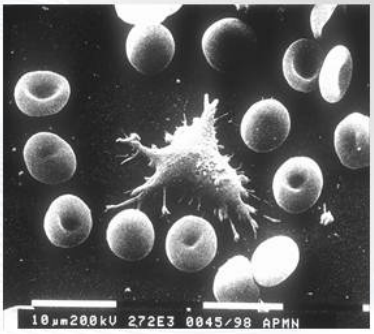


1995 Whole blood pyrogen test
2003 and 2004 Validated
2006 Validity statement
2009 Accepted Eur. Pharmacopoeia and with limitations by ICCVAM / FDA
PyroDetect (Biotest)

Novel Pyrogen tests based on the human fever reaction

Exogenous Pyrogen

1. Whole Blood
2. Leukocytes
3. Cell lines
4. Cryopreserved blood cells



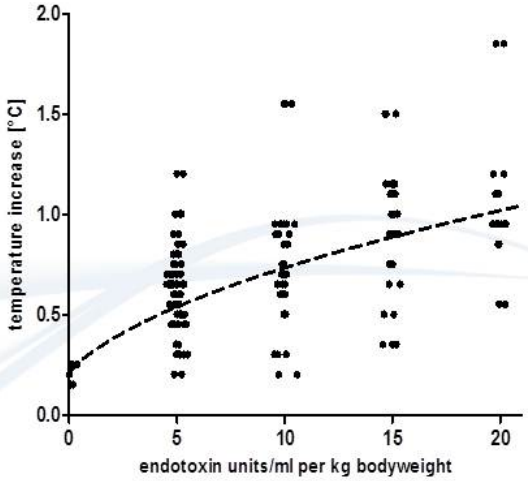
Endogenous Pyrogen

Fever *ELISA*

The rabbit test as reference

The temperature reaction of a sensitive strain (n = 171)

- limit temperature increase in Europe: 0.55° C
  - ➡ 5 EU/10 ml = 0.5 EU/ml defined as threshold
- 13 drugs with five contaminations each (0, 0.25, 0.5, 1 EU/ml)



challenging original study design

➡ (58% specific, 88% sensitive for rabbit)

Results – Predictive Capacity

Prediction Model 'PM0'

- non-pyrogenic: 0 EU/ml
- pyrogenic: 0.25, 0.5, 1 EU/ml

➡ 30 non pyrogenic and 120 pyrogenic samples

Test	Specificity	Sensitivity
PBMC	96.7%	100%
WBT A	92.6%	96.2%
WBT B	85.2%	99.0%
WBT fresh	85.7%	99.1%

Original Prediction Model

- non-pyrogenic: 0, 0.25 EU/ml
- pyrogenic: 0.5, 1 EU/ml

➡ 60 non pyrogenic and 90 pyrogenic samples

Test	Specificity	Sensitivity
PBMC	93.1%	77.6%
WBT A	98.8%	83.6%
WBT B	82.4%	89.1%
WBT fresh	97.4%	81.4%

Conclusion

The validated methods using cryopreserved cells

- **are an important standardisation**

  ➡ **widely applicable by overcoming limitations**

  - **availability of primary cells**
  - **exclusion of abnormal donors**
  - **test for infectious agents**

- **are reproducible and robust**
- **perform well in terms of predictive capacity**
- **should augment regulatory acceptance**

Adverse fever reactions by an infusion solution containing gelatine

negative Limulus Test as release criterion problematic batches recalled

samples blinded and sent to PEI

fever	rabbit	limulus	IL-1 pg/ml	IL-6 pg/ml	TNF pg/ml
-	-	-	8.5	28.0	28.2
+	+	-	142.6	654.4	67.6
+	-	-	421.5	9444.0	116.7

Non-endotoxin stimuli in the different tests

	pyrogen	LAL	blood-IL1	blood-IL6	PBMC-IL6	MM6-IL6	THP-1-Neo
Curdlan	-	+	-	-	-	-	-
Glucan-Barley	-	+	-	-	-	-	-
Glucan-Yeast	-	+	-	-	-	-	-
Glucan STD	-	+	-	-	-	-	-
Lipid A	-	+	-	-	-	-	-
PHA-E	-	+	-	-	-	-	-
PHA-L	+	+	+	+	+	+	+
Zymosan	-	+	-	-	-	-	-
LPS	+	+	+	+	+	+	+
LTA	+	-	+	+	+	+	+

New challenges

Patient safety: non-endotoxin pyrogens

New expression systems in gene technology
(Gram-positive bacteria, insect and fungal cells)

Medical devices

Cellular therapies

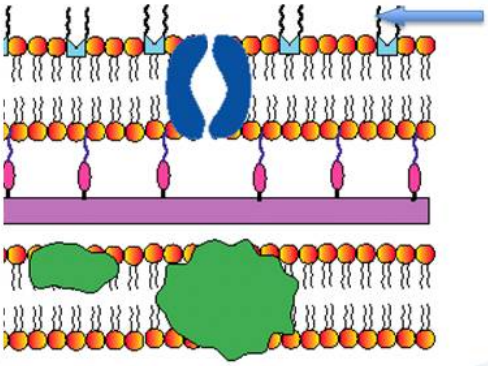
Environmental pyrogens

ICCVAM Recommendations: Future Studies May 2008

1. Both endotoxin-spiked and non-endotoxin spiked samples should be included. Non-endotoxin standards should be characterized prior to their use in any study, if possible.
2. … Good Laboratory Practice.
3. … products that have intrinsic pro-inflammatory properties …
4. Optimally, a study that includes 3-way parallel testing, with the *in vitro* assays being compared to the RPT and the BET, should be conducted to allow for a comprehensive evaluation of the relevance and comparative performance of these test methods. These studies may be conducted with historical RPT data provided that the same substances (i.e., same lot) are tested in each method. Based on ethical and scientific rationale, any *in vivo* testing should be limited to those studies that will fill existing data gaps.
5. Test substances that better represent all categories of sample types (e.g., pharmaceuticals, biologicals, and medical devices) intended for testing by the methods should be included.

Bacterial pyrogens

Gram-negative bacterial cell wall

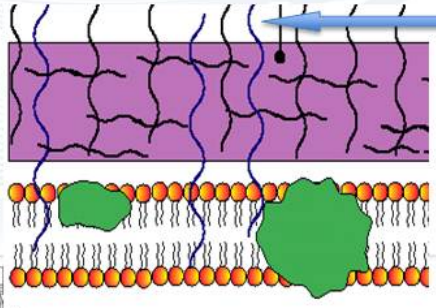


LPS

(Lipopoly-saccharide)

Medline publ. ~ 50 000

Gram-positive bacterial cell wall



LTA

(Lipoteichoic acid)

Medline publ. ~ 1 000

LTA is a Gram-positive endotoxin

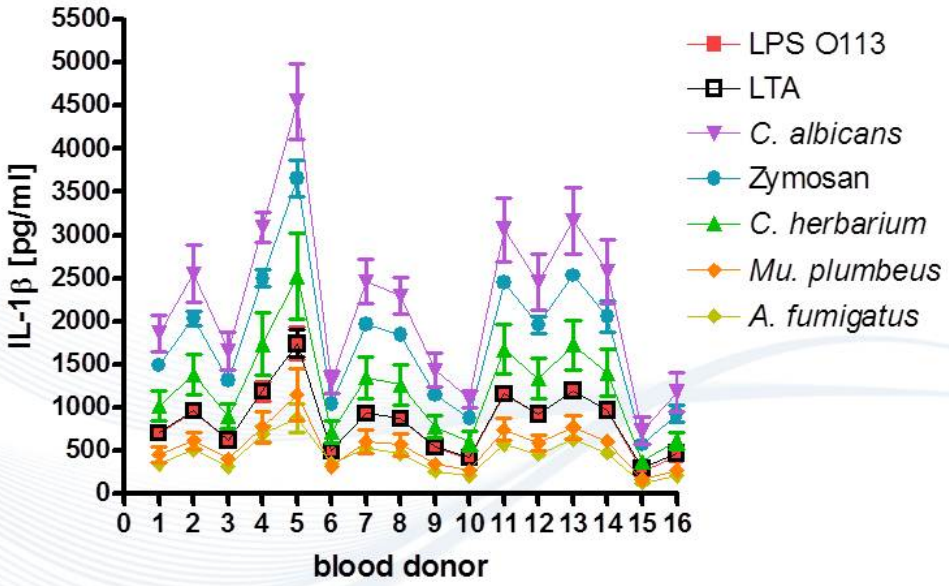
Contra	Pro
inactive when purified	similar structure
LPS contamination	conserved principle
synthetic LTA inactive	immunostimulatory
less potent than LPS	
No specific inhibitor	

LTA is a Gram-positive endotoxin

Contra

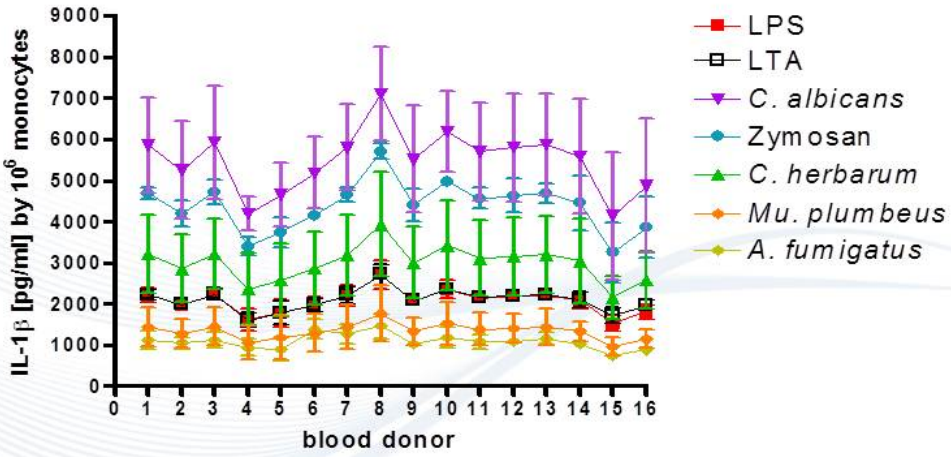
~~inactive when purified~~	Morath 2001, J. Exp. Med.
~~LPS contamination~~	Morath 2001, Inf. Immun.
~~synthetic LTA inactive~~	Morath 2002, J. Exp. Med. Deininger 2003, J. Immunol. Deininger 2007, Clin. Vacc. Imm.
~~less potent than LPS~~	von Aulock 2003, Immunobiol Deininger 2008, Immunobiol
~~No specific inhibitor~~	Draing 2008, Eur. J. Immunol.

Comparison of different donors



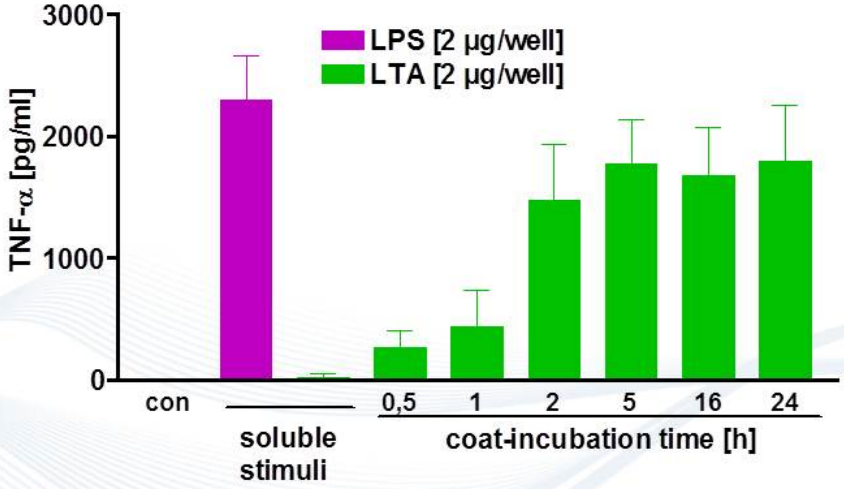
Different absolute but similar relative response of donors

Relative response of donors



Differences caused mainly by different monocyte counts

Surface bound LTA is more potent



Only 1% of LTA binds to surface

Presentation of LTA on a surface increases 1000fold its inflammatory potency

Deininger, Immunobiol. 2008

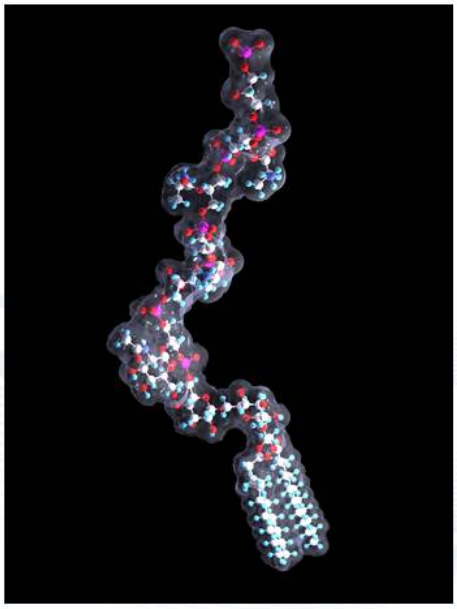
Systematic review following Evidence-based Medicine as to the fulfillment of Koch/Dale criteria for the major candidate structures inducing cytokine release in human monocyte / macrophages

Koch / Dale	LTA	PGN	Lipoproteins
Presence in bacteria	+++	ubiquitous	-
Synthesis inhibition abrogates cytokines	+	not possible	+
Isolated substance induces cytokines	+++	++	++
Block of substance	+	-	-

1-5 publications: +; 6-10 publications: ++; >10 publications: +++

Rockel, 2011, submitted

Why is the nature of the Gram-positive endotoxin 50 years after identification of the Gram-negative one still under discussion?



- **LTA instability**
- **Presentation effect (potency)**
- **Differences mouse to human**
- **Not complete endotoxin (IFNγ)**
- **Limited commercial availability**
- **No specific test comparable to LAL**

Toward a follow-up validation

- Set up a validation management group
- Design the validation study
- Choice of test materials and non-endotoxin pyrogens
- GLP?
- Suggested:
  - LTA and lysates of Gram-pos bacteria
  - Rabbit testing only for spike materials
  - Limited reproducibility re-assessment